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Development of Sensory Neurons in the Absence of NGF/TrkA Signaling In Vivo

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Summary

The neurotrophin survival dependence of peripheral neurons in vitro is regulated by the proapoptotic BCL-2 homolog BAX. To study peripheral neuron development in the absence of neurotrophin signaling, we have generated mice that are double null for BAX and nerve growth factor (NGF), and BAX and the NGF receptor TrkA. All dorsal root ganglion (DRG) neurons that normally die in the absence of NGF/TrkA signaling survive if BAX is also eliminated. These neurons extend axons through the dorsal roots and collateral branches into the dorsal horn. In contrast, superficial cutaneous innervation is absent. Furthermore, rescued sensory neurons fail to express biochemical markers characteristic of the nociceptive phenotype. These findings establish that NGF/TrkA signaling regulates peripheral target field innervation and is required for the full phenotypic differentiation of sensory neurons.

Introduction

The neurotrophins are a family of polypeptide growth factors that act via specific receptor tyrosine kinases (the Trk family) to regulate the development and functional properties of neurons throughout the nervous system (for review, see Snider, 1994; Lewin and Barde, 1996; Reichardt and Farinas, 1997). Studies over the past decade in mice with targeted deletions of the neurotrophin and *trk* genes have demonstrated that neurotrophin/Trk signaling is essential for the survival of most classes of peripheral neurons during embryonic development. However, due to this absolute survival dependence, these "knockout" models have not been informative as to the role of the neurotrophins in regulating axon growth and neuronal differentiation.

Particularly well characterized in terms of neurotrophin dependencies are the sensory neurons of the dorsal root ganglia (DRG). These neurons can be segregated according to sensory modality, with the largest population being the nociceptors, which require nerve growth factor (NGF) for survival (for review, see Snider and Silos-Santiago, 1996). In mice with targeted deletions of the *NGF* or *trkA* genes, ~80% of the normal complement of DRG neurons are lost (Crowley et al., 1994; Smeyne et al., 1994; Silos-Santiago et al., 1995). Roughly half of these are small- and medium-sized neurons that express TrkA as well as calcitonin gene-related peptide (CGRP) in maturity. In addition, a group of nociceptive neurons that downregulate TrkA postnatally and upregulate the glial cell line–derived neurotrophic factor (GDNF) receptor Ret also require NGF in embryonic life (Silos-Santiago et al., 1995; Molliver et al., 1997; for review, see Snider and McMahon, 1998).

Although survival regulation of nociceptors by NGF/TrkA signaling has been amply demonstrated in gene-targeted mice, suspected regulation of other important developmental events by NGF has yet to be proven. For example, despite profound pharmacological effects of NGF on sensory axon growth and the knowledge that NGF is synthesized in developing skin, whether NGF is a critical mediator in the establishment of skin innervation is unknown (Scott, 1992; see also Kennedy and Tessier-Lavigne, 1995). Indeed, experimental evidence to date has suggested that early outgrowth of sensory axons in vivo is neurotrophin independent (Davies et al., 1987; Davies, 1994; O'Conner and Tessier-Lavigne, 1999). Furthermore, a variety of other neuronal growth factors are synthesized in skin that may mediate axonal arborizations there (Farinas et al., 1996; Rice at al., 1998; Fundin et al., 1999; see also Maina et al., 1997). Similarly, although NGF has well-characterized and robust pharmacological effects on peptide synthesis, whether NGF plays any role in the acquisition of biochemical phenotypes of TrkA-expressing neurons is unknown (Verge et al., 1995; Hall et al., 1997; Molliver and Snider, 1997; Tonra and Mendell, 1998).

Recent advances in the understanding of programmed cell death have led to the identification of molecules associated with the intracellular signaling pathways underlying apoptosis (for review, see Merry and Korsmeyer, 1997; Pettmann and Henderson, 1998). Of particular importance to the peripheral nervous system is the proapoptotic BCL-2 homolog BAX. In BAX-deficient mice, sympathetic and DRG neurons survive indefinitely in vitro in the absence of NGF (Deckwerth et al., 1996; Lentz et al., 1999). Furthermore, naturally occurring cell death is virtually eliminated in peripheral ganglia of BAX-deficient mice during embryonic life (White et al., 1998). Gross development of the nervous system, however, appears normal, and no neurological deficits in these mice have yet been reported (Deckwerth et al., 1996; White et al. 1998). These latter observations suggest that BAX itself is not required for the regulation of developmental processes other than neuronal survival (see also Lentz et al., 1999). BAX-deficient mice may therefore provide a unique system in which to separate the survival requirement for neurotrophins from their effects on target field innervation and differentiation in vivo.

To characterize the development of DRG neurons in the absence of NGF/TrkA signaling, we have crossed *Bax* null mice with *trkA* null and *NGF* null mice. We show here that all NGF/TrkA-dependent neurons survive in

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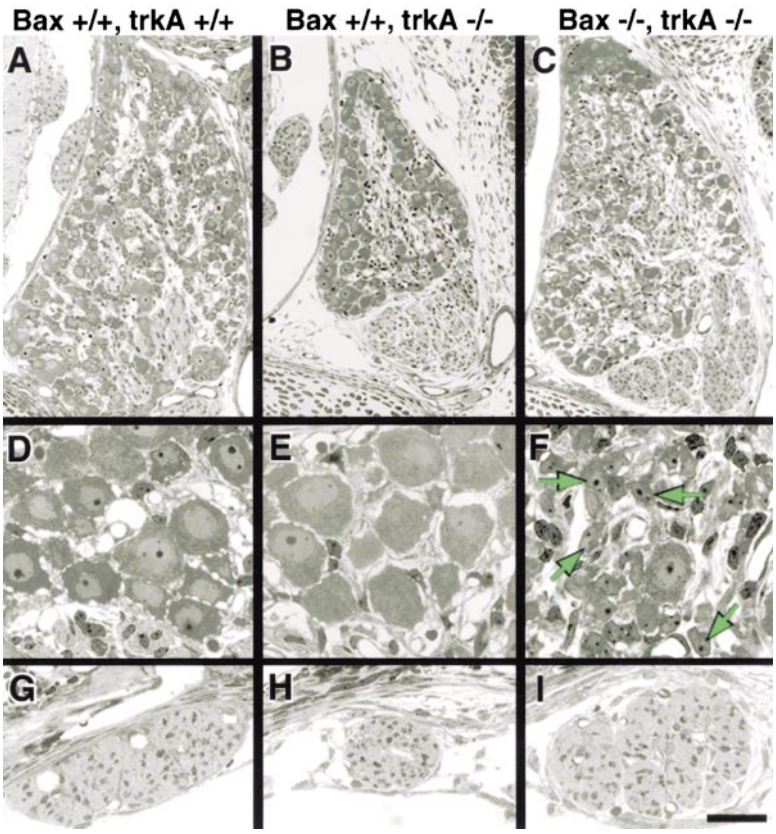

Figure 1. Small Cells Survive in *Bax*$^{-/-}$/*trkA*$^{-/-}$ DRGs

(A–C) Semithin sections of L4 DRGs at P0 reveal that the *Bax*$^{+/+}$/*trkA*$^{-/-}$ DRG is considerably reduced in size compared with wild-type DRG due primarily to the loss of medium- and small-sized neurons, which depend on NGF/TrkA signaling for survival. In contrast, the overall size of the L4 DRG from the *Bax*$^{-/-}$/*trkA*$^{-/-}$ mouse is not visibly different from that of the wild-type DRG.

(D–F) Higher power photomicrographs. In the *Bax*$^{+/+}$/*trkA*$^{-/-}$ DRG, only large neurons survive. In contrast, the DRG of *Bax*$^{-/-}$/*trkA*$^{-/-}$ mice contains numerous small neurons (arrows). These neurons are atrophic in comparison to the medium and small neurons of the wild-type DRG.

(G–I) Semithin sections of dorsal roots from wild-type and mutant mice. The dorsal root from the *Bax*$^{+/+}$/*trkA*$^{-/-}$ mouse is considerably reduced in size relative to that of the wild type. In contrast, the dorsal root from the *Bax*$^{-/-}$/*trkA*$^{-/-}$ mouse is larger than that of the wild type due to the increase in the total number of dorsal root axons.

Scale bar, 80 μm (A–C); 25 μm (D–F); and 40 μm (G–I).

the double null mice and extend axons into the dorsal roots. Many of these axons extend collaterals into the superficial laminae of the spinal cord. Peripheral innervation, however, is deficient, and sensory neurons never develop appropriate biochemical phenotypes. Our results establish a requirement for NGF in the development of peripheral innervation as well as an essential role in sensory neuron differentiation.

Results

BAX-Deficient DRG Neurons Survive in the Absence of NGF/TrkA Signaling in Vivo

To assess whether sensory neurons deprived of NGF/TrkA signaling could survive in vivo if BAX were also deleted, *Bax*$^{-/-}$/*trkA*$^{-/-}$ mice were generated by crossing heterozygotes from each line. The *Bax/trkA* double nulls were physically indistinguishable from their wild-type littermates at birth. These mice, however, did not survive beyond the first postnatal week. In addition, like the *trkA*$^{-/-}$ mice, the double nulls did not vocalize in response to tail pinch, indicating the absence of nociceptive input.

Figures 1A–1F show low- and high-power semithin sections of lumbar 4 (L4) DRGs from postnatal day 0 (P0) wild-type and mutant mice. L4 DRGs from *Bax*$^{+/+}$/*trkA*$^{-/-}$ mice were markedly reduced in size compared with wild type, as expected given the known dependence of DRG neuron number on NGF/TrkA signaling (Smeyne et al., 1994; Silos-Santiago et al., 1995). In contrast, ganglia from the *Bax*$^{-/-}$/*trkA*$^{-/-}$ mice were grossly normal in size, suggesting that sensory neurons survived in the absence of NGF/TrkA signaling.

Examination at higher power confirmed the rescue of sensory neurons in the *Bax/trkA* double null mice. Neuronal profiles of various sizes were found in wild-type DRG, reflecting the presence of small TrkA-dependent and large TrkC-dependent neurons within the ganglion (Figures 1D–1F). Predominantly, large neuronal profiles were found in the *Bax*$^{+/+}$/*trkA*$^{-/-}$ mutants due to the elimination of the small- and medium-sized NGF/TrkA-dependent populations. In the *Bax/trkA* double nulls, however, numerous small neuronal profiles (Figure 1F, arrows) were present in every section examined, indicating that at least a portion of the NGF/TrkA-dependent population survived. Although these were considerably smaller than were the small neurons in the wild-type DRG, they could be confidently distinguished from glia due to the presence of distinct round nuclei (Figure 1F, arrowheads).

To verify that these small, "rescued" cells were in fact viable neurons, we stained for expression of the intermediate filament protein peripherin (Figures 2A–2C), which is found in peripheral neurons but not in nonneuronal cells. Peripherin immunoreactivity was broadly distributed in the wild-type ganglia, and the vast majority of cells were labeled. Large surviving neurons were clearly peripherin positive in the *trkA*$^{-/-}$ DRG. In the *Bax/trkA* double null mice, numerous small peripherin-labeled cells (Figure 2C, arrows) were found in the center of the DRG in addition to the large neurons distributed around the periphery. These findings demonstrate that not only do DRG neurons survive in the absence of NGF/TrkA signaling but that they also express neuron-specific markers.

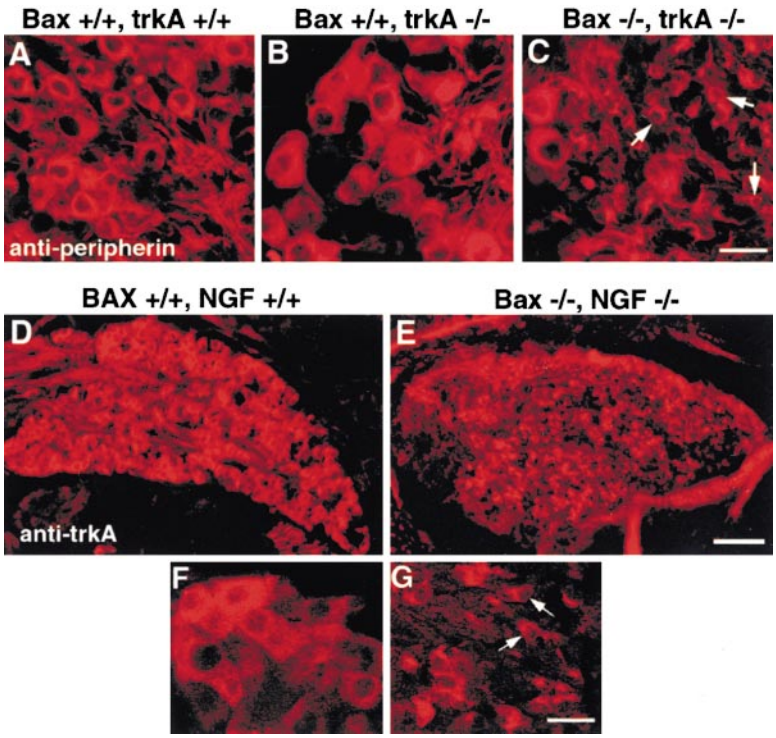

Figure 2. Rescued Small Cells Express Neuronal Markers

(A–C) Photomicrographs of peripherin immunoreactivity in DRG neurons from mice of the indicated genotypes. In *Bax*$^{+/+}$*/trkA*$^{-/-}$ DRGs, only large cells are stained, consistent with the loss of all medium- and small-sized neurons in these mice. In the *Bax*$^{-/-}$*/trkA*$^{-/-}$ DRG, many small cells (arrows), in addition to a number of large neurons, express peripherin. These peripherin-labeled small cells are considerably smaller than the small peripherin-labeled cells in the wild-type DRG. Scale bar, 25 μm (A–C).

(D and E) TrkA immunoreactivity in DRGs from mice of the indicated genotypes. Note the comparable sizes of the wild-type and double null ganglia (compare [D] and [E]). Surviving neurons in the interior of the ganglion in double nulls clearly express TrkA. Scale bar, 75 μm (D and E).

(F and G) Confocal images of TrkA protein expression. In the *Bax/NGF* double nulls, most cells express TrkA but are clearly atrophic (arrows) compared with TrkA-expressing cells in wild-type ganglia. Scale bar, 25 μm (F and G).

To assess the magnitude of neuronal survival in the *Bax/trkA* double nulls, L4 DRG neuron counts and dorsal root axon counts were performed (Table 1). Total dorsal root axon numbers were determined from sampling electron microscopy photomicrographs of dorsal root sections. Across all genotypes, there was a close correspondence between the DRG neuron counts and the dorsal root axon counts, thus validating the counting methods employed.

As expected, there was a massive loss of DRG neurons and dorsal root axons in the *Bax*$^{+/+}$*/trkA*$^{-/-}$ mice. In contrast, in the *Bax* null mice, the neuron and axon counts were considerably higher than in wild-type controls, regardless of the presence or absence of the *trkA* gene. L4 DRG neuron and dorsal root axon counts were increased by ~60% in the *Bax*$^{-/-}$*/trkA*$^{+/+}$ mice, presumably due to the elimination of naturally occurring cell death (see also White et al., 1998). Comparable increases were observed in *Bax*$^{-/-}$*/trkA*$^{-/-}$ mice, indicating that all neurons that require NGF/TrkA signaling during embryonic life are rescued from apoptosis if BAX is not present.

To establish our findings in mice of a known genetic background, *Bax*$^{-/-}$*/NGF*$^{-/-}$ double knockouts were also generated from crosses of single mutants maintained on a pure C57Bl/6 genetic background. Similar to the results from *Bax*$^{-/-}$*/trkA*$^{-/-}$ mice, we found that DRGs from *Bax*$^{-/-}$*/NGF*$^{-/-}$ mice were comparable in size to wild-type DRGs at equivalent levels (compare Figures 2D and 2E). Furthermore, in *Bax*$^{-/-}$*/NGF*$^{-/-}$ mice, many small peripherin-labeled neurons were also found in the DRG (data not shown). Not only did these small neurons express peripherin in the Bax/NGF double knockouts, but they also exhibited robust immunoreactivity for TrkA (Figures 2E and 2G), indicating that the NGF/TrkA-dependent population, specifically, survives in the absence of neurotrophin signaling. Thus, BAX-deficient, TrkA-expressing DRG neurons survive in the absence of NGF in the C57Bl/6 strain.

BAX-Deficient DRG Neurons Elaborate Spinal Collateral Branches in the Absence of NGF/TrkA Signaling

As indicated by the L4 dorsal root axon counts, the *Bax*$^{-/-}$*/trkA*$^{-/-}$ neurons elaborate axons through the dorsal roots toward the spinal cord. Indeed, the dorsal roots from the double null mice were larger than those of wild-type mice, reflecting the increase in the number of dorsal root axons (Figure 1, lower panels). To assess whether these sensory axons elaborated collateral branches into the dorsal horn of the spinal cord, we examined the distribution of peripherin in the spinal cord of P0 wild-type and mutant mice (Figures 3A–3E). In wild-type mice, peripherin-labeled axons were found extending in the

Table 1. Summary of L4 DRG Neuron Counts and L4 Dorsal Root Axon Counts in P0 *Bax/trkA* Crosses

	Bax$^{+/+}$, *trkA*$^{+/+}$	*Bax*$^{+/+}$, *trkA*$^{-/-}$	*Bax*$^{-/-}$, *trkA*$^{+/+}$	*Bax*$^{-/-}$, *trkA*$^{-/-}$
L4 DRG neurons	8,618 (100%)	884 (10%)	14,160 (164%)	13,791 (160%)
L4 DR axons	8,880	737	13,003	14,850

n = 5 for DRG neurons counts; n = 3 for dorsal root axon counts.

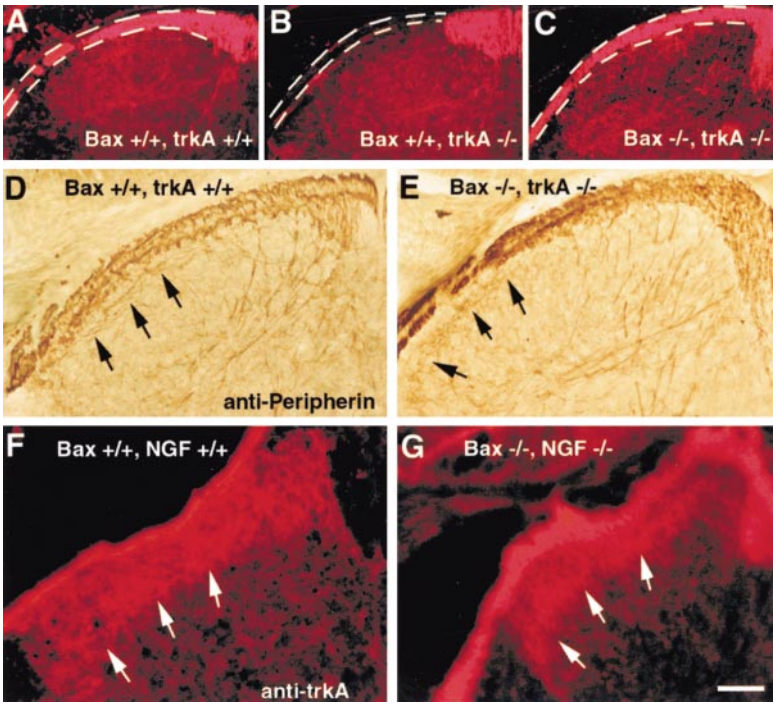

Figure 3. BAX-Deficient DRG Neurons Project to the Spinal Cord in the Absence of NGF/TrkA Signaling

(A–C) Immunofluorescent peripherin labeling of central projections in the dorsolateral funiculus from mice of the indicated genotypes. The boundary of the dorsolateral funiculus is demarcated by the dashed lines. In the *Bax*$^{-/-}$*/trkA*$^{-/-}$ mice, peripherin immunoreactivity is absent, consistent with the loss of NGF/TrkA-dependent DRG neurons and their central projections. In the *Bax*$^{-/-}$*/trkA*$^{-/-}$ mice, abundant peripherin immunoreactivity is apparent in the dorsolateral funiculus.

(D and E) Peripherin labeling of the innervation in the spinal cord dorsal horn. The pattern of innervation in *Bax*$^{-/-}$*/trkA*$^{-/-}$ mice is similar to that seen in *Bax*$^{+/+}$*/trkA*$^{+/+}$ mice. Arrows point to peripherin-labeled axon branching in lamina I of the superficial dorsal horn.

(F and G) TrkA immunoreactivity in the dorsal horn of *Bax*$^{+/+}$*/NGF*$^{+/+}$ and *Bax*$^{-/-}$*/NGF*$^{-/-}$ mice. TrkA labeling (arrows) is similarly distributed in the dorsal horns of both wild-type and double null mice. In both, the labeling is confined to the superficial laminae I and II. Scale bar, 50 μm (F and G).

dorsolateral funiculus (Figure 3A, dashed lines) and penetrating the superficial lamina of the dorsal horn (Figure 3D, arrows). No peripherin-immunoreactive axons were found in the dorsolateral funiculus of *Bax*$^{+/+}$*/trkA*$^{-/-}$ mice (Figure 3B), consistent with the loss of all small-caliber DRG axons. In contrast, peripherin-labeled axons were found extending through the dorsolateral funiculus (Figure 3C) and penetrating the superficial lamina of the dorsal horn in the *Bax*$^{-/-}$*/trkA*$^{-/-}$ mice (Figure 3E, arrows). Furthermore, the distribution and density of peripherin-labeled axons were indistinguishable from that observed in wild-type mice. These observations demonstrated that small sensory neurons retain their capacity to respond to appropriate spinal branching cues even in the absence of NGF/TrkA signaling.

Innervation of the dorsal horn of the spinal cord was also examined in the *Bax*$^{-/-}$*/NGF*$^{-/-}$ mice by immunostaining of TrkA-labeled axons (Figures 3F and 3G). In the wild-type spinal cord, TrkA labeling was confined to laminae I and II. Not only was the trkA-labeled innervation rescued in the *Bax*$^{-/-}$*/NGF*$^{-/-}$ mice, but also the distribution of TrkA axons in the superficial dorsal horn was similar to that observed in the wild-type mice. Thus, the NGF/TrkA-dependent neurons extend collateral branches and navigate to appropriate spinal target fields in the absence of NGF/TrkA signaling. Whether TrkA axons form synapses with appropriate subsets of spinal neurons in these mice has not yet been studied.

Superficial Cutaneous Innervation Is Absent in *Bax*$^{-/-}$*/trkA*$^{-/-}$ Mice

To determine if the surviving sensory neurons innervate their peripheral targets in the absence of NGF/TrkA signaling, we examined hindlimb skin and the mystacial pads of wild-type and mutant mice by staining for the panaxonal marker PGP 9.5 (protein gene product 9.5). PGP 9.5 staining was found in the major nerve trunks in the distal hindlimb of P0 wild-type mice (Figure 4A) and revealed the extensive collateral branching of axons in the dermis and epidermis (Figure 4B). In the *Bax*$^{+/+}$*/trkA*$^{-/-}$ mutants, axons in the major nerve trunks were stained due to the presence of non-trkA-dependent sensory axons (Figures 4A–4F). Collateral innervation to the upper dermis and epidermis was virtually absent, as expected (Figures 4C and 4D). In contrast to the situation in spinal cord, BAX deletion did not restore this superficial innervation of the skin (Figures 4E and 4F). Similar results were obtained in hindlimb sections stained with antibodies against the intermediate filament peripherin (data not shown). Furthermore, the failure of sensory axons to innervate the skin was apparent at embryonic stages of development. Thus, at embryonic day 15 (E15), we also noted the absence of PGP 9.5–labeled axons arborizing in the skin of the proximal hindlimb and ventral body surface of *Bax*$^{-/-}$*/trkA*$^{-/-}$ mice (data not shown).

Results were similar in *Bax/NGF* double null mice on a pure C57Bl/6 background. In the *Bax*$^{+/+}$*/NGF*$^{+/+}$ mice, numerous TrkA-positive axons extended in the main nerve trunk and arborized in the skin of the distal hindlimb (Figure 4G, arrows). In contrast, TrkA-labeled axons were not found in the distal hindlimb, and collateral branches were absent in the skin of the *Bax*$^{-/-}$*/NGF*$^{-/-}$ mice (Figure 4H). Thus, both the ligand, NGF, and the receptor, TrkA, are required for the development of superficial cutaneous innervation.

To assess axon projections through a major cutaneous nerve trunk, axons counts were performed in the saphenous (SA) (cutaneous) nerve at the level of electron microscopy (Table 2). Consistent with the loss of DRG neurons, ~90% of the axons in the SA nerve were lost in the *Bax*$^{+/+}$*/trkA*$^{-/-}$ mice. In the *Bax*$^{-/-}$*/trkA*$^{-/-}$ mice, ~86% of the axons were also missing. These counts

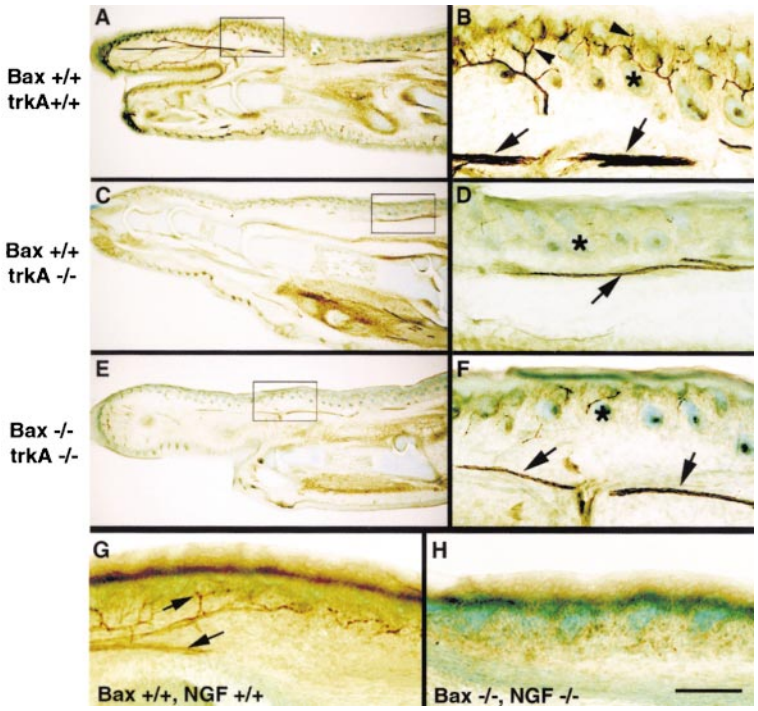

Figure 4. Cutaneous Innervation in the Hindlimb Is Absent at P0

(A–F) PGP 9.5 labeling in the distal hindlimb of wild-type and Bax/TrkA mutants. On the left are low-power photomicrographs (A, C, and E) of the distal limb and foot. PGP 9.5–labeled nerve trunks with extensive branches are seen in the wild-type hindlimb (arrows). Boxed areas correspond to the higher-power photomicrographs on the right (B, D, and F) showing innervation of the superficial skin. PGP 9.5–labeled nerve trunks (arrows) can be seen extending just deep to the skin in all three genotypes. In wild-type hindlimb, extensive axonal branching (arrowheads, [B]) is seen in the superficial skin. In contrast, in the $Bax^{+/+}/trkA^{-/-}$ and $Bax^{-/-}/trkA^{-/-}$ hindlimbs, arborization in the skin is substantially reduced. Asterisks denote hair follicles in the dermis.

(G and H) TrkA immunoreactivity in the hindlimb of $Bax^{+/+}/NGF^{+/+}$ and $Bax^{-/-}/NGF^{-/-}$ mice at P0. TrkA-labeled axons are found in the main nerve trunk, and branching is found in the skin. No TrkA labeling was detected in the $Bax^{-/-}/NGF^{-/-}$ distal hindlimb.

Scale bar, 450 μm (A, C, and E), and 100 μm (B, D, and F–H).

suggest that NGF/TrkA-dependent axons either never entered or could not be maintained in the major cutaneous nerve branches.

The absence of peripheral target innervation in the $Bax^{-/-}/trkA^{-/-}$ mice was not limited to DRG neurons, as similar deficiencies were observed in the sensory innervation of the mystacial pads arising from neurons in the trigeminal ganglion (Figure 5). Mystacial pads were analyzed with combined neurofilament and PGP 9.5 histochemistry, which labels all peripheral innervation. In wild-type whisker pad, numerous axons in the deep vibrissal nerve (Figure 5A, large arrow) and robust innervation and branching in the upper dermis and epidermis (Figure 5A, small arrows) were observed. The pattern was similar in $Bax^{-/-}/trkA^{+/+}$ mice, except for an apparent increase in axons in the vibrissal nerve and in the innervation of the upper dermis and epidermis (Figure 5B). In contrast, in the $Bax^{+/+}/trkA^{-/-}$ mice, only a few neurofilament- and PGP 9.5-labeled axons were found in the vibrissal nerve, and arborization in the superficial skin was absent (Figure 5C). The pattern and density of superficial innervation in the double nulls were similar to that observed in the mystacial pads of $Bax^{+/+}/trkA^{-/-}$ mice (Figure 5D). These results were in agreement with the observations in the hindlimb skin and suggest a requirement for NGF/TrkA signaling in the establishment of normal peripheral projections, even for neurons that extend axons over relatively short distances.

$Bax^{-/-}/trkA^{-/-}$ DRG Neurons Do Not Express Peptides

CGRP and substance P (SP) are distinct neurochemical markers of the nociceptive, TrkA-expressing DRG neurons (Verge et al., 1989; Molliver et al., 1995; Bennett et al., 1996). To determine whether NGF/TrkA signaling is required for peptide expression, we examined CGRP and SP immunoreactivity in the DRGs from wild-type and mutant mice at P0 (Figures 6A–6F). The presence of corresponding SP mRNA was evaluated by in situ hybridization (Figures 6G–6I). In wild-type DRG, numerous small- and medium-sized CGRP- and SP-immunoreactive neurons were identified. In addition, SP mRNA was abundant in these cells, as indicated by the robust hybridization signal. The pattern of peptide expression was similar in the DRGs of $Bax^{-/-}/trkA^{+/+}$ mice, demonstrating that BAX-deficient DRG neurons express peptides normally (data not shown). Peptide immunoreactivity and mRNA were absent in the DRGs of $Bax^{+/+}/trkA^{-/-}$ mice, consistent with the loss of NGF/TrkA-dependent neurons. Perhaps surprisingly, in the $Bax^{-/-}/trkA^{-/-}$ mice, no peptide immunoreactivity was observed in the DRG. Furthermore, SP mRNA expression was absent, indicating regulation at the transcriptional level. These results suggest a requirement for NGF/TrkA signaling for the initiation of peptide synthesis, although they do not reveal whether the effect is direct or indirect (see below).

Previous work from our laboratory demonstrated that

Table 2. Summary of SA Nerve Axon Counts in P0 *Bax/trkA* Crosses

	$Bax^{+/+}$, $trkA^{+/+}$	$Bax^{+/+}$, $trkA^{-/-}$	$Bax^{-/-}$, $trkA^{+/+}$	$Bax^{-/-}$, $trkA^{-/-}$
Axon count	2817 (100%)	294 (10%)	3737 (133%)	392 (14%)

n = 3 for each group.

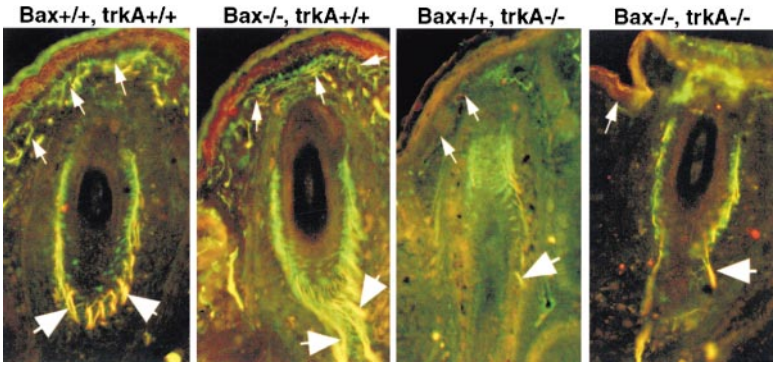

Figure 5. Cutaneous Innervation in the Mystacial Pads Is Absent at P0

Superimposed double immunofluorescence labeling of the innervation in whisker pads of P0 wild-type, *Bax*$^{-/-}$/*trkA*$^{+/+}$, *Bax*$^{+/+}$/*trkA*$^{-/-}$, and *Bax*$^{-/-}$/*trkA*$^{-/-}$ mice. Each image shows the innervation to a single whisker follicle and the overlying epidermis. The upper dermis and epidermis in the wild type (small arrows) have a rich innervation that is completely lacking in the *Bax*$^{+/+}$/*trkA*$^{-/-}$ and *Bax*$^{-/-}$/*trkA*$^{-/-}$ mice. Also, numerous labeled axons from the deep vibrissal nerve (large arrows) can be seen in the wild type. There is an apparent increase of labeled axons in the vibrissal nerve and epidermis of the *Bax*$^{-/-}$/*trkA*$^{+/+}$ mice. In contrast, few axons from the vibrissal nerve are seen in the whisker pads of *Bax*$^{+/+}$/*trkA*$^{-/-}$ and *Bax*$^{-/-}$/*trkA*$^{-/-}$ mice. Neurofilament 200 (NF200) is revealed by Cy3 (red), and PGP 9.5 by Cy2 (green). Structures double labeled with anti–neurofilament 200 and anti–PGP 9.5 are yellow.

a subpopulation of nonpeptidergic nociceptive neurons express the GDNF receptor Ret. These neurons express TrkA in embryonic life but downregulate TrkA postnatally (Silos-Santiago et al., 1995; Molliver and Snider, 1997; Molliver et al., 1997). To assess whether the onset of Ret expression is dependent on NGF/TrkA signaling, we stained P0 DRG sections for Ret expression (Figures 6J–6L). In wild-type DRG, numerous Ret-immunopositive neurons were visible at P0. As expected, few Ret-immunoreactive neurons were found in *Bax*$^{+/+}$/*trkA*$^{-/-}$ DRG due to the loss of all TrkA-expressing cells by E15. Similar to the results with CGRP and SP, Ret immunoreactivity was absent in the *Bax*$^{-/-}$/*trkA*$^{-/-}$ mutants. This result suggests that TrkA expression and NGF/TrkA signaling are prerequisites for Ret expression. Thus, acquisition of biochemical phenotypes of both the peptidergic and the nonpeptidergic populations of nociceptors appears to be regulated by NGF signaling through TrkA.

CGRP Expression Is Absent in NGF-Deprived, BAX-Deficient DRG Explants

To resolve whether the lack of peptide staining in *Bax*$^{-/-}$/*trkA*$^{-/-}$ DRGs in vivo was due to the requirement for NGF or a consequence of failure to innervate targets, we cultured E13.5 *Bax*$^{-/-}$ DRG with and without NGF. By starting at E13.5, we could evaluate CGRP initiation independent of target contact and be confident that all nociceptive cells had been born (Lawson and Biscoe, 1979; Kitao et al., 1996). Explants were cultured for 3.5 days, until they had reached the equivalent in vivo age of E17, the earliest stage at which peptide expression is detected in vivo (Molliver and Snider, 1997; see also Marti et al., 1987; Hall et al., 1997; Jackman and Fitzgerald, 2000). After fixation, we examined explants for SMI 31 (green), CGRP (red), and TrkA (red) immunoreactivity (Figure 7). Glial cells were unlabeled.

In wild-type explants in which neurons were dependent on appropriate neurotrophins for survival, CGRP was expressed in ~50% of cells when maintained in the presence of NGF (data not shown). Expression of CGRP by 50% of NGF-dependent DRG neurons is consistent with the in vivo expression pattern at this developmental stage, E17 (Molliver and Snider, 1997), and indicates that CGRP expression develops in an appropriate time frame and proportion in vitro (see also Hall et al., 1997).

Cells were stained with antibodies to TrkA under all experimental conditions to establish that some degree of nociceptive phenotypic differentiation had occurred. In wild-type explants cultured in the presence of NGF, all cells expressed TrkA (data not shown). Results in *Bax*$^{-/-}$ explants maintained in NGF showed TrkA labeling in ~70%–80% of cells (Figures 7A and 7C). Non-TrkA-expressing neurons, which were presumably neurotrophin-3 (NT-3) dependent, were markedly atrophic (Figure 7C, box). In explants cultured in the absence of NGF (Figures 7B and 7D), TrkA expression was also detected in 70%–80% neurons. As expected, all neurons in this condition were atrophic (Figure 7D).

In the presence of NGF (Figures 7E and 7G), neurons distributed throughout the explant expressed CGRP in a pattern similar to that observed in vivo. Detailed cell counts determined that mean expression of CGRP in the NGF condition was 41% ± 2.7%, also consistent with previous in vivo data (Molliver and Snider, 1997). At high magnification (Figure 7G), the boxed area shows small negative cells (presumably NT-3-dependent, as in Figure 7C). Importantly, however, there were numerous larger negative cells (Figure 7G, asterisks). This finding parallels in vivo observations that only a subset of TrkA-positive DRG neurons express CGRP at late embryonic and early postnatal stages (see Molliver et al., 1997, and references therein).

In the absence of NGF, CGRP expression (Figures 7F and 7H) was restricted to a few isolated cells, even though most of these cells expressed TrkA. These data imply that initiation of peptide synthesis for most TrkA-expressing cells requires direct action of NGF, although this may not be the only inductive signal. Cells that did express CGRP tended to be larger in size, suggesting that they were responding to some trophic signal, possibly found in the serum. It is tempting to speculate that these cells were expressing the α transcript of CGRP, which is found in larger DRG neurons in vivo (Noguchi et al., 1990).

Discussion

Our study establishes that BAX is required for neuronal apoptosis triggered by neurotrophin deprivation in vivo. Surviving neurons showed selective developmental deficits. Axon extension into the dorsal root and collateralization into the spinal cord proceeded normally. In contrast, peripheral innervation and peptide expression

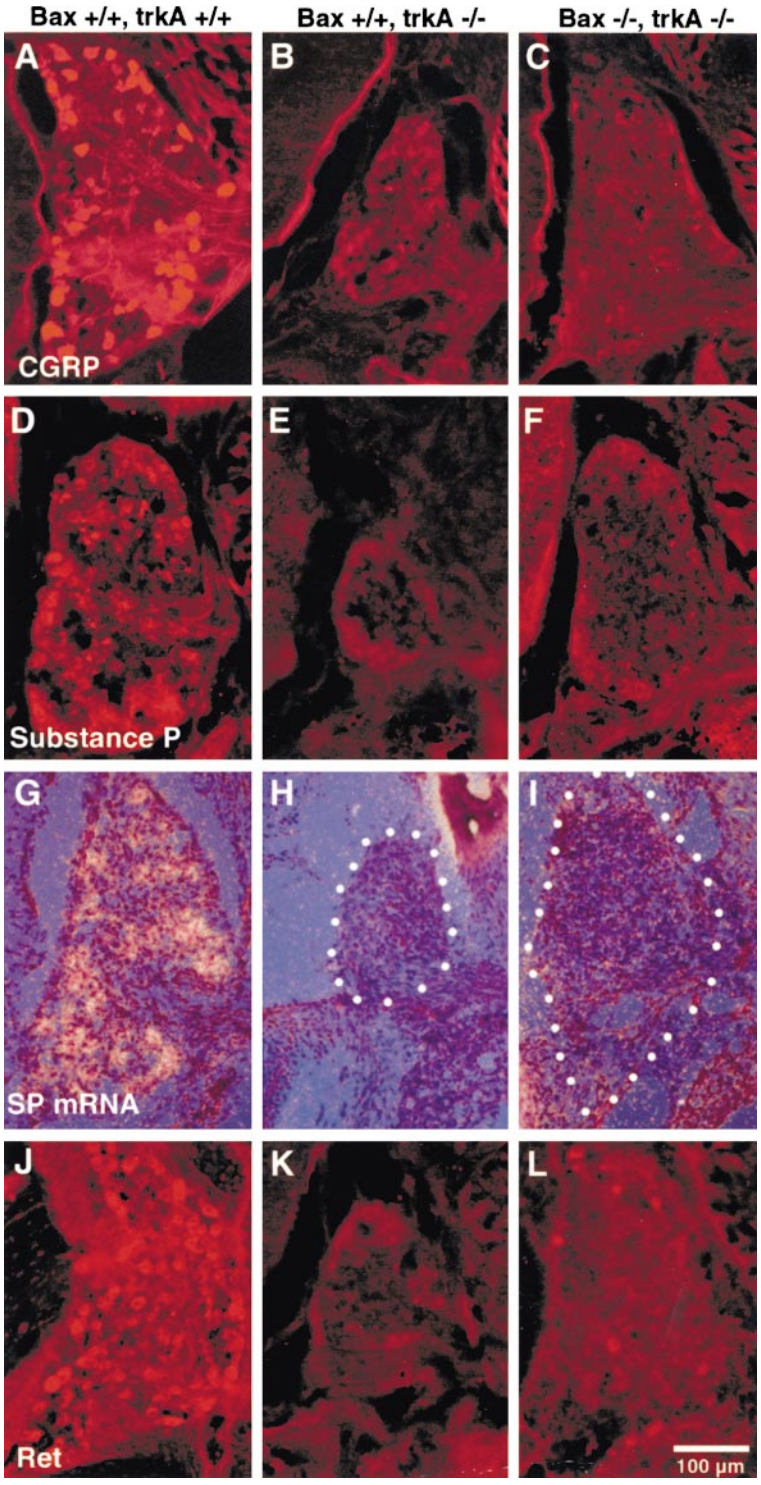

Figure 6. BAX-Deficient DRG Neurons Fail to Express Multiple Phenotypic Markers Characteristic of Nociceptive Neurons in the Absence of NGF/TrkA Signaling

(A–C) CGRP immunoreactivity.
(D–F) SP immunoreactivity.
(G–I) SP mRNA.
(J–L) Ret immunoreactivity in DRGs from mice of the indicated genotypes.

Numerous CGRP- and SP-immunoreactive neurons are found in the wild-type DRG, and abundant SP mRNA in the wild-type DRG corresponds with the presence of SP peptide. CGRP- and SP-immunoreactive neurons are not found in the $Bax^{+/+}/trkA^{-/-}$ DRGs. Although DRG neurons survive in the $Bax^{-/-}/trkA^{-/-}$ mice, these neurons do not exhibit immunoreactivity for CGRP and SP and do not express SP mRNA.

Numerous Ret-immunoreactive neurons are distributed in the wild-type DRG at P0. Ret immunoreactivity is absent in the $Bax^{+/+}/trkA^{-/-}$ DRG. In the $Bax^{-/-}/trkA^{-/-}$ DRG, the surviving neurons do not exhibit Ret labeling.

failed to develop. Our results prove a long suspected role for NGF in regulating skin innervation and establish a previously unsuspected requirement for NGF in biochemical differentiation.

Apoptosis Induced by Neurotrophin Deprivation In Vivo Requires BAX

There is no doubt that DRG neurons survived the deprivation of neurotrophin signaling in $Bax^{-/-}/trkA^{-/-}$ mice. First, the ganglia were almost of normal size, in contrast to the markedly reduced size of the ganglia in *trkA* nulls. Second, although many neurons were atrophic, neurons and glia could be readily distinguished in semithin sections in the double nulls, allowing verification of neuronal rescue. Third, the small rescued cells expressed the neuron-specific intermediate filament protein peripherin. Finally, dorsal root axon counts revealed a virtual one-to-one correspondence with cell counts in both

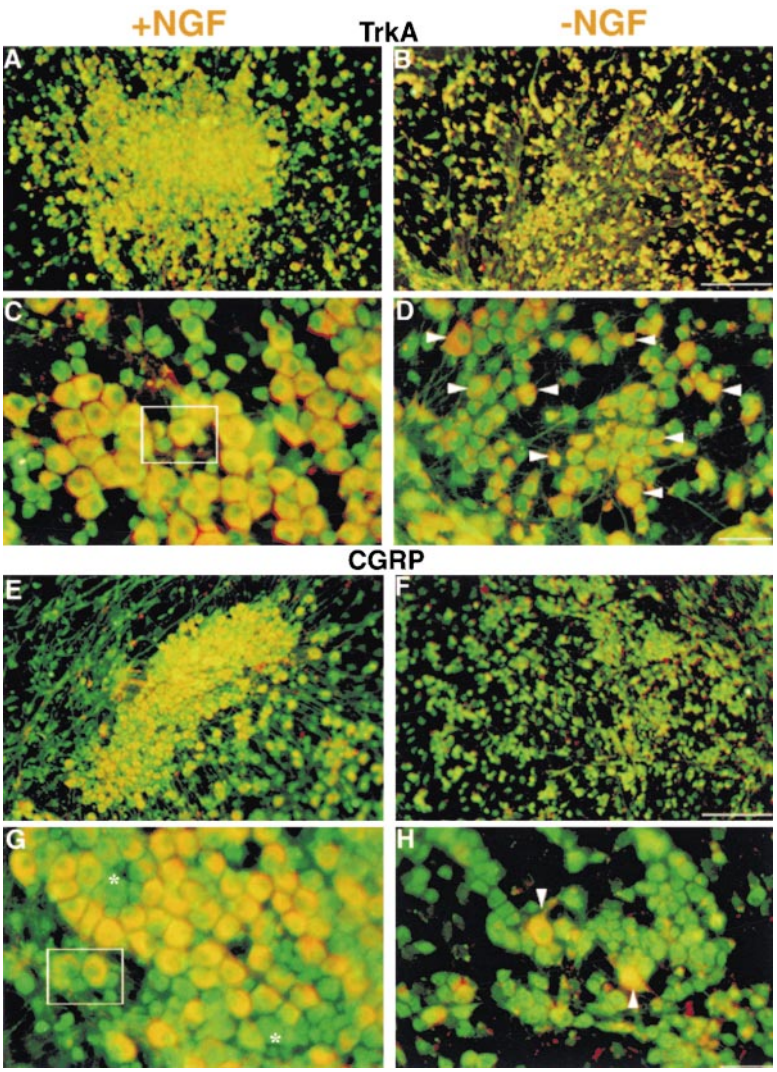

Figure 7. CGRP Expression in Embryonic *Bax*$^{-/-}$ DRG Explants Requires NGF

(A–D) Immunolabeling of SMI 31 (green) and TrkA (red) in DRG explants. In the presence of NGF, 70%–80% of cells express TrkA. Note that the TrkA-negative cells in the boxed area (C) are smaller than are the positive cells, as they are nonresponsive to NGF and atrophic. TrkA expression is maintained (arrowheads, [D]) without NGF, but all cells are atrophic. Scale bar, 100 μm (A and B), and 25 μm (C and D).

(E–H) Immunolabeling of SMI 31 (green) and CGRP (red) in DRG explants. In NGF (E and G), CGRP expression is ~40%. Boxed area shows small negative cells, while asterisks denote large negative cells. In (F) and (H), the absence of NGF results in loss of CGRP expression, except for a few isolated positive cells (arrowheads, [H]). Scale bar, 100 μm (E and F), and 25 μm (G and H).

wild-type and mutant mice. It is clear that the total number of DRG neurons in the *Bax*$^{-/-}$/*trkA*$^{-/-}$ mice is even greater than in wild type and is comparable to that found in *Bax*$^{-/-}$ mice. This is consistent with our prior observation that naturally occurring cell death in DRGs is prevented in vivo by the elimination of BAX (White et al., 1998).

Qualitatively similar results were obtained in the *Bax*$^{-/-}$/*NGF*$^{-/-}$ mice. DRGs from these mice were of comparable size to the wild-type ganglia, and a large number of small atrophic neurons were present in the central regions of the *Bax*$^{-/-}$/*NGF*$^{-/-}$ DRGs. Indeed, these atrophic cells expressed TrkA, thereby conclusively demonstrating that the TrkA population survived NGF deprivation. These results are particularly important because both the NGF mutant and BAX mutant strains used for breeding had been maintained on a pure C57Bl/6 background. Thus, our result that deletion of BAX confers neurotrophin-independent survival in vivo is confirmed in mice of known genetic composition. Finally, it appears that our results will generalize to sensory neurons dependent on other neurotrophin family members. Preliminary results show that TrkC-expressing sensory neurons survive in the absence of NT-3 if BAX is also deleted (W. D. Snider et al., 1999, Soc. Neurosci., abstract). Taken together, our results establish that apoptosis induced by deprivation of neurotrophin signaling during development in vivo requires BAX.

Collateral Branching in the Spinal Cord Develops Independently of NGF/TrkA Signaling

Presumptive nociceptive DRG neurons in the *Bax*$^{-/-}$/*trkA*$^{-/-}$ mice extended central axonal projections toward the spinal cord in a manner that appeared remarkably normal under light level histological analysis. We found that the dorsal root axon counts were comparable to the DRG neuron counts in the *Bax*$^{-/-}$/*trkA*$^{-/-}$ mice, demonstrating that all rescued neurons extend a central process. Additionally, immunohistochemistry demonstrated that these axons elaborated collateral branches into the spinal cord gray matter. Finally, at least some axons projected to their normal target fields in laminae I and II, suggesting that they retained the capacity to respond to guidance and branching cues. Again, this result was confirmed in *Bax*$^{-/-}$/*NGF*$^{-/-}$ mice in which

TrkA-labeled axons were present in the dorsal horn and confined to laminae I and II.

Our results demonstrate that NGF is not involved in regulating the collateral branching of sensory axons into the dorsal horn. This finding is consistent with the fact that NGF is not present at significant levels in spinal cord (Leibrock et al., 1989). Similarly, a number of experiments have suggested that NT-3 is not involved in the initiation of spinal collateral axon branching, even though NT-3 is expressed in cord at high levels at early developmental stages (Ozaki and Snider, 1997; Wright et al., 1997; Sharma and Frank, 1998). Finally, it has been shown in vitro that peripheral, target-derived factors do not influence laminar specific innervation of the dorsal horn (Redmond et al., 1997; see also Yamagata and Sanes, 1995).

The recent discovery that Slit2, a member of the Slit family of *Drosophila* midline guidance homologs, possesses a branch-inducing activity for NGF-dependent DRG neurons in vitro may provide an explanation for the ability of sensory neurons to collateralize in the spinal cord (Wang et al., 1999). Vertebrate members of the Robo family, which are receptors for the Slits, are expressed in the DRG during the time period when DRG axons are penetrating into spinal gray matter (Brose et al., 1999; Wang et al., 1999). Whatever the nature of the molecular cues that initiate collateral branching, our results demonstrate that the capacity of the central process of DRG neurons to respond is not regulated by NGF.

An important caveat about the central projections of rescued sensory neurons is that we have no information about functional connectivity. Whether sensory axons arborize in the appropriate topographic regions of the dorsal horn or form synapses with appropriate partners is unclear. Indeed, several recent studies demonstrate the importance of connections with peripheral targets to the establishment of functional central synapses (Wenner and Frank, 1995; also see Lin et al., 1998, and references therein). For example, proprioceptive axons deprived of contact with peripheral muscle spindles extend collateral branches into the spinal cord but fail to form synaptic contacts with motor neurons (E. Frank et al., 1999, Soc. Neurosci., abstract). Altogether, our results suggest that the complex process of branching, navigation, and synaptogenesis of sensory collaterals is multifactorial in relation to control by the periphery. The early stages of axon extension and collateral branching appear to be independent of peripheral control, whereas formation of appropriate synaptic contacts may require a peripheral influence.

Peripheral Cutaneous Innervation Requires NGF/TrkA Signaling

In contrast to the central projections, cutaneous arborizations of peripheral sensory axons were absent in the *Bax*$^{-/-}$/*trkA*$^{-/-}$ mice. Thus, the current findings establish that NGF/TrkA signaling is required for innervation of the skin. Furthermore, even though brain-derived neurotrophic factor, NT-3, NT-4, and GDNF are present in developing skin (see Reichardt and Farinas, 1997; Rice et al., 1998; Fundin et al., 1999, and references therein), these other growth factors apparently do not mediate this arborization or compensate for the loss of NGF/TrkA signaling. Although we cannot completely exclude the possibility that the peripheral projections developed normally at an earlier developmental stage, the presence of demonstrable abnormalities at E15 suggests that appropriate arborizations never form rather than not being maintained in the absence of NGF/TrkA signaling.

Surprisingly, axon counts in the purely cutaneous SA nerve in *Bax*$^{-/-}$/*trkA*$^{-/-}$ were markedly reduced to a level comparable to that found in the *Bax*$^{+/+}$/*trkA*$^{-/-}$ mice. Consistent with these observations, TrkA-labeled axons were not found in the major nerve trunks of the hindlimb of *Bax*$^{-/-}$/*NGF*$^{-/-}$ mice. Taken together, these results suggest that peripheral cutaneous axons fail to extend into the hindlimb in the absence of NGF/TrkA signaling. These findings are surprising, as axon extension from peripheral ganglia is thought to be neurotrophin independent (Lumsden and Davies, 1983; Davies et al., 1987; Davies, 1994, 1996). Indeed, a provocative recent study has suggested that nonneurotrophin trophic factors derived from intermediate targets may support axon growth until the arrival of axons in their target fields (Wang and Tessier-Lavigne, 1999). Hence, one might have expected that axon growth in the major nerve trunks would be normal and that only arborization in the skin would fail. We suspect that the massive elongation of axons required to keep pace with limb growth is dependent on NGF (see Lentz et al., 1999). In this scenario, neurons failing to acquire NGF at the onset of NGF synthesis in the hindlimb at E11 (White et al., 1996) would be unable to extend axons normally thereafter. We should point out, however, that our results in *Bax*$^{-/-}$/*NGF*$^{-/-}$ mice do not exclude the possibility that NT-3, acting through TrkA, may mediate peripheral axon growth to some degree. Indeed, NT-3 is synthesized in the mesenchyme along the path of growing peripheral axons (Farinas et al., 1996; Patapoutian et al., 1999), and it has been suggested that TrkA is a mediator of NT-3 signaling in developing trigeminal ganglion neurons in vivo (Huang et al., 1999).

NGF may also function as a chemoattractant at the earliest stages of skin innervation and/or as a branching factor at later developmental stages. Although a chemoattractant role for NGF has long been hypothesized, it has never been convincingly demonstrated as a normal developmental mechanism (Tessier-Lavigne and Placzek, 1991; O'Conner and Tessier-Lavigne, 1999). The details of initial axon growth and arborization in mice deprived of NGF/TrkA signaling will require a robust marker specifically for the TrkA population. Studies of axonal projections in *Bax/trkA* double null mice in which the gene for the axonal marker τLacZ has been inserted into the *trkA* locus (Patapoutian and Reichardt, 1999, Gordon Conference on Neurotrophins) are underway and should resolve the role of NGF at the earliest stages of sensory axon outgrowth.

NGF/TrkA Signaling Is Required for the Acquisition of Functional Biochemical Phenotypes

Although the *Bax*$^{-/-}$/*trkA*$^{-/-}$ DRG neurons survive in the absence of NGF/TrkA signaling, these neurons are clearly abnormal in that they fail to acquire biochemical phenotypes characteristic of nociceptive neurons. We

show that these neurons do not express the neuropeptides CGRP and SP or the GDNF receptor tyrosine kinase Ret (see Molliver and Snider, 1997). The absence of peptide immunoreactivity and the failure to upregulate Ret in *Bax*$^{-/-}$/*trkA*$^{-/-}$ DRGs raise a number of possibilities regarding the role of NGF/TrkA signaling in the acquisition of specific sensory neuron phenotypes.

In light of our observation that NGF/TrkA-dependent axons fail to innervate their peripheral target fields in the skin, one possibility is that NGF/TrkA signaling may not be directly involved in initiating peptide synthesis. Rather, NGF may facilitate the interaction between DRG neurons and other target-derived factors, which, in turn, regulate the onset of peptide expression. One way to distinguish between these possibilities is to evaluate peptide expression in vitro under conditions in which sensory neurons are removed from target-derived influences. Prior in vitro work has indicated that CGRP develops intrinsically in DRG neurons in a time course comparable to that observed in vivo (Hall et al., 1997). A potential role for NGF could not be addressed in these previous studies because it was not possible to culture embryonic DRG neurons without appropriate neurotrophins.

Our in vitro data with BAX-deficient explants show that CGRP does not develop in the absence of NGF, even when all nociceptive neurons survived, as evidenced by TrkA protein expression. This result suggests a critical requirement for NGF in the differentiation of neurons into the peptidergic phenotype. Indeed, virtually all neurons that express the nociceptive peptides CGRP and SP also express TrkA and are responsive to NGF (Verge et al., 1989; Averill et al., 1995; Molliver et al., 1995). In both developing and mature DRG neurons, NGF powerfully regulates the levels of expression of SP and CGRP (Kessler and Black, 1980; Lindsay and Harmar, 1989; Verge et al., 1995). Despite these effects on regulation of peptide synthesis at later developmental stages and in maturity, available evidence has argued against a direct role for NGF/TrkA signaling in initiating peptide expression. Thus, acquisition of the peptidergic phenotype in vitro over a delayed time frame has raised the possibility of cell-autonomous regulation (Hall et al., 1997). Furthermore, although postnatal manipulation of NGF affects levels of peptide expression, it does not alter the percentage of CGRP-positive neurons within the NGF/TrkA-dependant population (Verge et al., 1995; Molliver and Snider, 1997; Tonra and Mendell, 1998). However, none of these studies addressed the requirement of NGF for peptide acquisition due to an inability to deprive sensory neurons of NGF prior to the onset of peptide expression. Our results demonstrate that when neurons are kept alive in vivo or in vitro in the absence of NGF/TrkA signaling, they do not initiate expression of neuropeptides.

The simplest explanation for the requirement of NGF for peptide expression would be a direct effect of NGF activation of TrkA on the initiation of peptide synthesis. A role for NGF in early differentiation has not previously been established, but it does not seem surprising. Peptides are not expressed until well after sensory neurons express TrkA and the onset of NGF expression in the skin (White et al., 1996; Molliver and Snider, 1997; Farinas et al., 1998; Jackman and Fitzgerald, 2000). Furthermore, our finding that NGF is necessary for peptidergic differentiation is consistent with the known consequences of NGF-activated signal transduction. Thus, TrkA, in response to NGF, mediates activation of mitogen-activated protein (MAP) kinase and the transcription factor cAMP response element–binding protein (CREB), which in turn regulates transcription of a large number of genes (Ginty et al., 1994; Xing et al., 1998). The peptide genes preprotachykinin and calcitonin/CGRP both contain CRE binding sites, enabling them to bind activated CREB (Broad et al., 1989; Kageyama et al., 1991; Watson and Latchman, 1995). In addition, the CGRP enhancer is strongly activated by the MAP kinase kinase, MEK-1 (Durham and Russo, 1998). Presumably, these pathways are functional at the earliest developmental stages and could mediate NGF/TrkA regulation of peptidergic differentiation.

It is important to emphasize that the presence of a defined pathway leading from NGF to sensory neuron peptidergic differentiation does not exclude a requirement for other factors. In fact, our cultures contained serum, which might have provided necessary cofactors. Furthermore, recent work published in abstract form identifies bone morphogenetic proteins as potential regulators of CGRP expression in developing sensory neurons (X. Ai and A. K. Hall, 1998, Soc. Neurosci., abstract). Finally, in maturity, the dramatic effects of axotomy and inflammation on peptide expression are not explained solely by changes in levels of NGF (Lewin et al., 1994; Woolf et al., 1994; see also Zigmond and Sun, 1997). Although these studies suggest that other factors may be involved, our work demonstrates that NGF/TrkA signaling is a necessary component of sensory neuron biochemical differentiation.

Conclusion

In sum, we have established conditions in which peripheral neurons survive in vivo in the absence of neurotrophin/Trk signaling. Neurotrophin-deprived neurons exhibit selective developmental deficits. Peripheral target field innervation and full-phenotypic differentiation are shown to require neurotrophin/Trk signaling. More broadly, we expect these mice to be a definitive tool for global analyses of neurotrophin-regulated gene expression during development.

Experimental Procedures

Breeding and Genotyping of *Bax/trkA* and *Bax/NGF* Double Nulls

The generation and characterization of *Bax*$^{-/-}$ mice have been previously described (Knudson et al., 1995; Deckwerth et al., 1996). *Bax*$^{+/-}$ mice were crossed with *trkA*$^{+/-}$ mice (Smeyne et al., 1994) to generate *Bax*$^{+/-}$/*trkA*$^{+/-}$ offspring. The *Bax/trkA* double heterozygotes were then mated to generate the *Bax*$^{-/-}$/*trkA*$^{-/-}$ mice. Because the mice used for the *Bax/trkA* crosses were maintained on a mixed genetic background, we also generated *Bax/NGF* double null mice from stocks maintained on a pure C57Bl/6 background. The C57Bl/6 *Bax*$^{+/-}$ mice were a generous gift from Dr. Stan Korsmeyer, and the C57Bl/6 *NGF*$^{+/-}$ were a generous gift from Dr. Heidi Philips and Genentech. Both genotypes had been maintained on the C57Bl/6 background for more than 15 generations. All animals were genotyped for normal and mutant alleles by tail DNA PCR.

Immunohistochemistry

P0 mouse pups were anesthetized and perfused with 4% paraformaldehyde. Tissue sections were cut on a cryostat at 18 μm tissue thickness for immunofluorescence and directly thawed onto glass slides. For DAB staining, sections were cut at 60 μm thickness and collected in phosphate-buffered saline (PBS). Sections were incubated in primary antibodies against CGRP (1:500, Peninsula Laboratories, Belmont, CA), SP (1:1000, Diasorin, Minneapolis, MN), neurofilament 200 (1:800, Chemicon, Temecula, CA), PGP 9.5 (1:1000, Accurate Biochemicals, Westbury, NY, and UltraClone, Wellow, UK), peripherin (1:300, Chemicon, Temecula, CA), TrkA (1:1000, from Dr. Louis Reichardt), or Ret (1:1000, from Dr. Qiao Yan, Amgen, Thousand Oaks, CA). Primary antibody incubations were carried out overnight at room temperature. All antibodies were diluted in 0.1 M PBS containing 1.5% normal goat serum and 0.3% Triton X-100. The following day, sections were washed and incubated in Cy2- or Cy3-conjugated antibodies (1:200, Jackson) or biotinylated secondary antibodies and processed for visualization using standard protocols.

In Situ Hybridization

In situ hybridization was performed on fresh frozen sections following established in situ hybridization protocols (see Wright and Snider, 1995, for a detailed protocol). RNA probes were transcribed from linearized plasmids containing a 560 bp cDNA encoding preprotachykinin. Transcription was carried out in the presence of 90 μCi [^{33}P]UTP (Amersham, Arlington Heights, IL) using either T7 or SP6 polymerases for antisense or sense probes, respectively. Sections were hybridized with either antisense or sense riboprobes (1×10^6 cpm/slide) diluted in hybridization mixture, and slides were hybridized overnight at 55°C. Subsequently, the slides were put through a series of rinses in sodium chloride citrate at 37°C and treated with RNase for 30 min at 45°C (RNase A, 40 μg/ml). Slides were emulsified in Kodak NTB-2 and stored at 4°C for 2 weeks before developing.

Tissue Processing for Light Microscopy

Anesthetized P0 mice were perfused with 2.5% gluteraldehyde/2% paraformaldehyde in 0.1 M cacodylate buffer. The lumbar spinal column and hindlimbs were excised, postfixed for 1 hr in 1% OsO_4, and embedded in plastic (Eponate 12). The L4 DRG (the ganglion associated with the thickest of the three spinal nerves giving rise to the sciatic nerve) was serially sectioned at 1 μm thickness and stained with 1% toluidine blue. The SA nerve (a major cutaneous nerve located at the medial side of hindlimb) was cross-sectioned at the level of the knee and examined in toluidine blue staining.

Neuron Counts

Sections of L4 DRGs were examined at 63× magnification at 60 μm intervals throughout the ganglion length. The number of neuronal nuclei visible in each section was counted and summed. The length of nuclei was then measured in a series of serial sections, taking a random sample of 50 nuclei. The total number of nuclei counted in the sample was multiplied by 60 (the sample interval) and divided by the mean nuclear length to give the total number of neurons in the entire DRG (Wright et al., 1997).

Nerve Fiber Counts

The L4 dorsal (sensory) spinal roots and the SA nerve were examined for the number of nerve fibers in plastic-embedded material. Transverse sections of dorsal roots and SA nerves of newborn mutants and their wild-type littermates were obtained on an ultramicrotome at a thickness of 90 nm. The sections were stained with lead citrate and uranyl acetate and examined by electron microscopy. The total number of nerve fibers (both myelinated and unmyelinated) in the L4 dorsal roots and SA nerves of mutant and wild-type newborns was determined from electron micrographs taken at 5000×. Statistical differences between mutant and wild-type values were examined with a Student's t test.

Explant Culture and Immunocytochemistry

Bax$^{+/-}$ male and female mice were bred to produce *Bax*$^{+/+}$, *Bax*$^{+/-}$, and *Bax*$^{-/-}$ offspring. The plug date was designated E0. Whole embryos were harvested from sodium pentobarbital–overdosed mothers at E13 under sterile conditions. Embryos were collected in ice-cold L15 medium supplemented with 5% heat-inactivated (HI) horse serum. Tails were used as a source of DNA to determine the genotype of embryos by PCR. DRGs from the entire rostrocaudal extent of the spinal cord were dissected and collected in minimal essential medium (Life Technologies, Gaithersberg, MD) containing 5% HI fetal bovine serum (Summit, Fort Collins, CO), 2 mm L-glutamine, 10 μM 5-fluoro-2′-deoxyuridine (Sigma, St. Louis, MO), and 1× penicillin/streptomycin. DRGs were stored overnight at 4°C while awaiting genotypes.

The following morning, DRG explants were plated on autoclaved glass coverslips (Thomas Scientific) and coated overnight with a mixture of poly-D-lysine (0.1 mg/ml, Sigma) and laminin (4 ng/ml, Collaborative Biomedical Products) in 24-well sterile culture plates (Fisher Scientific). Explants were maintained in the medium described above. Some cultures were supplemented with NGF (50 ng/ml) at the time of plating.

Cultures were maintained for 3.5 days and then fixed in methanol (10 min) followed by 4% paraformaldehyde (20 min). Explants were washed in PBS and incubated in blocking buffer consisting of 1.5% normal goat serum and 0.3% Triton X-100 in 0.1 M PBS for 1 hr at room temperature. Primary antibodies against phosphorylated neurofilaments H and M (SMI 31, 1:500, Sternberger Monoclonals, Baltimore, MD) and either CGRP (1:900, Peninsula Laboratories, Belmont, CA) or TrkA (1:1000, Dr. Louis Reichardt) were added and incubated for 2.5 hr at room temperature. Wells were rinsed multiple times in PBS and incubated in Cy2-conjugated goat anti-mouse immunoglobulin G (IgG) (1:100, Jackson) and Cy3-conjugated goat anti-rabbit IgG (1:200, Jackson) for 4 hr at room temperature. Explants were dehydrated through a series of ethanol washes, and coverslips were mounted on slides with Clearium (Surgipath) before visualization under FITC and rhodamine epifluorescence.

Each experiment was repeated with embryos from five separate matings. All SMI 31– and CGRP-immunoreactive cells were counted in 12 explants (derived from four experiments). CGRP-immunoreactive cells were expressed as a percentage of total cells ± SEM.

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